IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Fabrice Le Gall et al.	Art Unit: 1644
Application No. 10/527,346	Confirmation No.: 7228
Filed: September 23, 2005	Examiner: SKELDING, Zachary S.
For: HUMAN CD3-ANTIBODY WITH IMMUNOSUPPRESSIVE PROPERTIES	Attorney Docket: 03528.0146.PCUS00

RULE 132 DECLARATION OF DR. MELVYN LITTLE

I, Melvyn Little, Ph.D., Chief Scientific Officer at Affimed Therapeutics AG, hereby declare as follows:

1. I received a Ph.D. degree in biochemistry from University College of North Wales – Bangor, United Kingdom. After postdoctoral work on the mechanism of action of estradiol at the Max-Planck-Institute for Cell Biology in Wilhelmshaven, Germany, I joined the scientific staff of the German Cancer Research Center (DKFZ) in Heidelberg and habilitated at the Faculty of Biology, University Heidelberg. In 1990, I became head of the "Recombinant Antibodies" research group at the DKFZ.

2. I founded Affimed Therapeutics AG in 2000, which is the assignee of the above-referenced patent application. I currently serve as Chief Scientific Officer (CSO) at Affimed Therapeutics AG.

3. I am a co-inventor of the invention claimed in the above-referenced patent application.

4. I have reviewed the Office Action for the above-referenced patent application, in which the Examiner objected that in the Rule 123 Declaration submitted January 7, 2010 the claimed invention was not compared with a divalent OKT3 derived anti-CD3 antibody lacking an Fc domain, such as OKT3 derived $F(ab')_2$.

5. I supervised the following scientific experiment under item A), which demonstrates the superior property of the bivalent antibody claimed in the above-referenced

patent application over a bivalent F(ab´)$_2$ fragment. In particular, the following experiment compared the effects on T cell proliferation of an anti-CD3 diabody (i.e. a humanized anti-CD3 diabody (=scFv$_6$) according to the above-referenced patent application), which is devoid of constant domains, and an anti-CD3 F(ab´)$_2$ fragment derived from the anti CD3 IgG OKT3..

Further, under item B) the results obtained in the experiment were compared with the results reported for an anti-CD3 F(ab´)$_2$ fragment derived from the anti CD3 IgG OKT3 in Woodle et al. Transplantation 52, 354-360 (1991).

A) Proliferation of human PBMC in the presence of anti-human CD3 antibodies

1) Production of OKT3 F(ab´)$_2$

Materials and Methods

The OKT3 F(ab´)$_2$ was prepared from the murine IgG$_{2a}$ anti-CD3 OKT3 by pepsin digestion. For this purpose the Pierce F(ab')$_2$ preparation kit was used (Pierce 44988, Thermo Fisher Scientific Inc., Bonn, Germany). The sample was prepared following the instructions of the supplier.

The OKT3 F(ab´)$_2$ was analysed by HPLC size exclusion chromatography on a calibrated Superdex 200 HR10/300 GL column GE Healthcare, 17-5175-01, Freiburg, Germany). The column was mounted on a HPLC-Ultimate 3000 system (Dionex GmbH, Idstein, Germany). The chromatogram result was analyzed using Chromeleon software version 6.80.

Results

HPLC size exclusion chromatography of the murine IgG$_{2a}$ anti-CD3 OKT3 (Fig. 1) and of the OKT3 F(ab´)$_2$ (Fig.2) sample preparations are shown below. The chromatogram of figure 1, corresponding to the monoclonal antibody OKT3, showed that only a single symmetric peak eluting at 23.44 minutes is visible. In the case of the OKT3 F(ab´)$_2$, Fig. 2, a single symmetric peak eluting at 25.86 minutes is visible. This result showed clearly that the OKT3 F(ab´)$_2$ preparation did not contain any remaining full length antibody and could be used for direct comparison with the full length anti-CD3 OKT3.

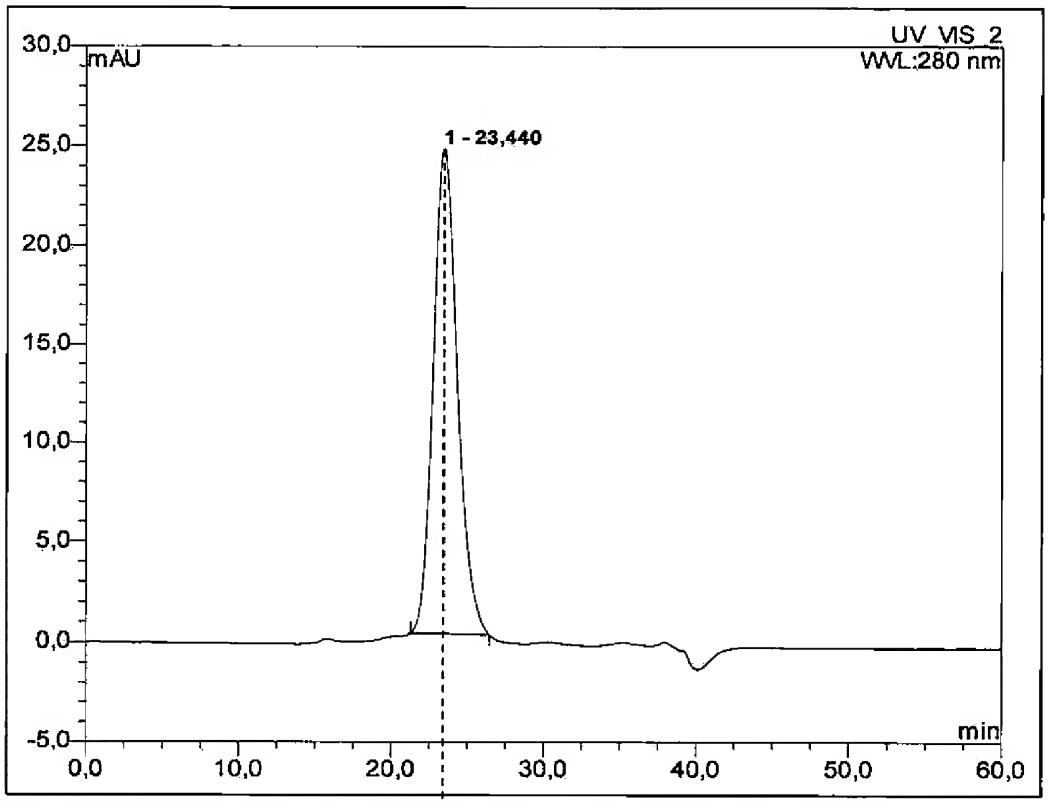

Fig 1. Size exclusion chromatogram of the murine IgG_{2a} anti-CD3 OKT3

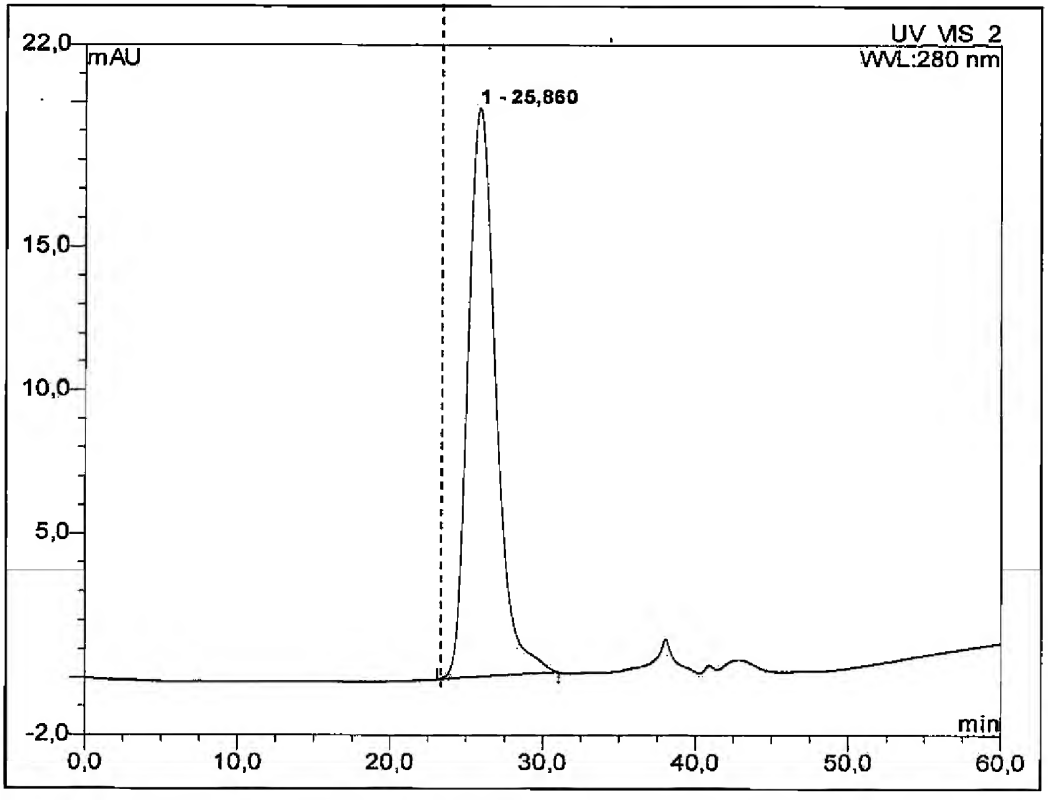

Fig 2. Size exclusion chromatogram of the OKT3 $F(ab')_2$

2) Proliferation of human PBMC in the presence of anti-human CD3 antibodies

Materials and Methods

To determine whether the bivalent diabody ($scFv_6$) or the bivalent $F(ab')_2$ fragments stimulate freshly isolated peripheral blood mononuclear cells (PBMC) to proliferate, a MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test was performed on cultures of human PBMC in the presence of increasing concentrations of anti-CD3 diabody derived from the murine anti CD3 mAb UCHT1, highly purified OKT3 $F(ab')_2$ fragments, buffer (20 mM Tris, pH 7.5) and, as a positive control, anti-CD3 IgG OKT3.

PBMCs were isolated from heparinized peripheral blood of a healthy volunteer by density gradient centrifugation. The blood sample was diluted with a two-fold volume of PBS (Gibco), layered on a cushion of Histopaque-1077 (Sigma) and centrifuged at 800 g for 25 min. PBMC located in the interface were collected and washed 3 times with PBS before they were resuspended in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine and 100 IU/ml penicillin G sodium and 100 µg/mL streptomycin sulfate (herein referred to as RPMI medium; all components from Invitrogen) and used in the proliferation assay.

2×10^5 PBMC were seeded in individual wells of a flat-bottom microplate together with increasing concentrations of the indicated antibodies or buffers in a total volume of 160 µL/well. After 5 days of incubation in a humidified incubator with 5% CO_2 at 37°C 24 µL/well dye solution (Promega) was added and the plate was incubated for a further 4 hours until a strong color development was observed. In order to solubilize the formazan crystals, 160 µL/well of the stop/solubilization solution (Promega) was added to the wells, mixed and incubated for an additional 2 hours. The absorbance of the samples was measured at 570 nm with a microplate reader (Victor 3, Perkin Elmer) and the absorbance at the reference wavelength at 650 nm was subtracted. Mean and standard deviation of triplicates were plotted in a diagram using the GraphPad Prism software.

Results

The results are shown in Fig. 3 below. While the anti-CD3 diabody ($scFv_6$) according to the claimed invention did not induce any proliferation at all concentrations tested, the OKT3 $F(ab')_2$ fragments showed an increasing stimulation to the proliferation of PBMCs at antibody concentrations above 1 µg/mL. The OKT3 IgG antibody demonstrated high mitogenic activity. The dye absorbance at the antibody concentration of 10 µg/mL is 0.00 for the diabody according to the claimed invention, 0.12 for the OKT3 $F(ab')_2$ fragment and 0.66 for the whole OKT3 antibody (the starting values without antibody have been subtracted from the

values measured at 10 µg/mL).

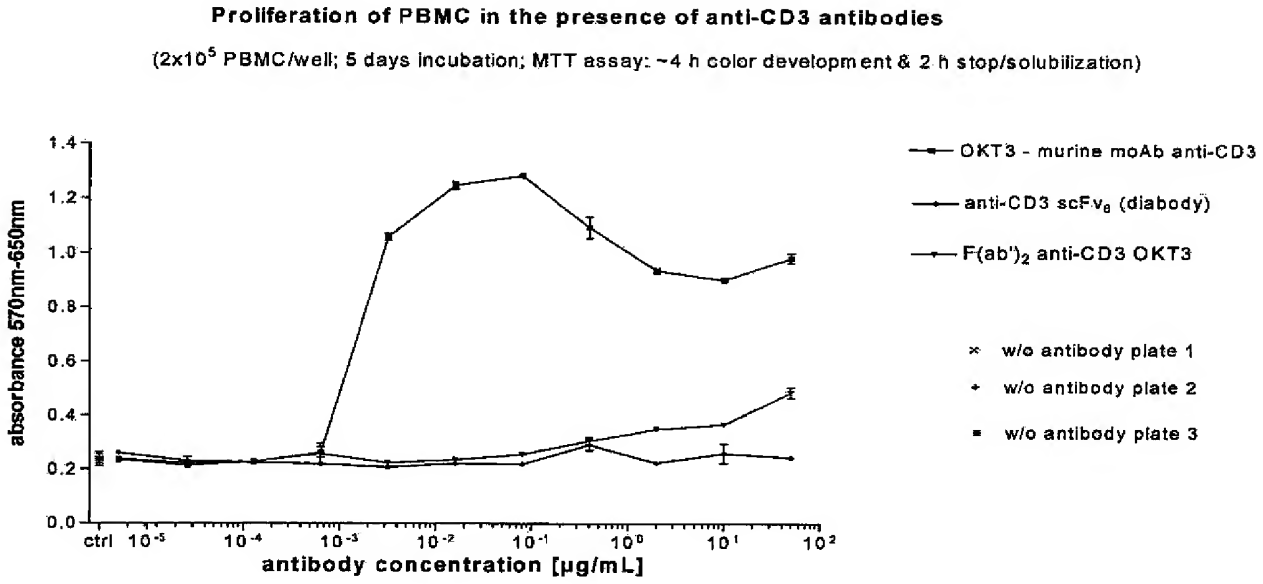

Fig. 3: Proliferation of human PBMC in the presence of anti-human CD3 antibodies. 2 x 10^5 PBMC were seeded in individual wells of a 96-well micro plate in the presence of the indicated concentrations of murine anti-CD3 antibody OKT3, humanized diabody $scFv_6$, and highly purified OKT3 F(ab')$_2$. After 5 days incubation at 37°C in a humidified incubator with 5% CO_2 the relative amount of living cells was determined with an MTT assay. Mean and SD absorbance values of triplicates are plotted in the diagram ("x", " +" and "*" indicate the values of the control samples in the absence of antibodies in each of the three tests).

B) Comparison with the results of Woodle et al. (Transplantation 52, 354-360 (1991))

Woodle et al. tested whole monoclonal antibody and F(ab')$_2$ fragments of OKT3 for their immune-activating effects on PBMCs. 1 x 10^5 cells per well were incubated with OKT3 full length antibody or highly purified OKT3 F(ab')2 fragments for three days and then one extra day in the presence of 3H-Thymidine (in round-bottomed micro plates) (see page 355, 3rd paragraph, indicated with "proliferation assays"). The amount of incorporated thymidine was measured in a liquid scintillation counter.

Woodle et al. show the effect of highly purified anti-CD3 F(ab')$_2$ fragments on the proliferation of PBMCs in figures 3 and 4. Whereas Fig. 3 shows the effect of increasing concentrations of F(ab')$_2$, Fig. 4 shows the effect of F(ab')$_2$ at two reference concentrations of 10µg/mL and 1ng/mL.

At antibody concentrations of 10 µg/mL (=10^4 ng/mL) the amounts of incorporated radioactive thymidine for OKT3 F(ab')$_2$ were approximately 16000 cpm in Fig. 3 and approximately 32000 cpm in Fig. 4; while the amounts for whole OKT3 antibody were

approximately 90000 cpm in Fig 3 and 75000 cpm in Fig. 4.

Therefore, the data of Woodle et al. show a significant stimulation of PBMC proliferation by OKT3 F(ab´)$_2$ at an antibody concentration of 10 µg/mL.

The results of the experiment under item A) and the data of Woodle et al. in item B) are compared in Table 1 below:

	CPM 3H-Thymidine	Absorbance of converted dye
whole OKT3 Fig.3	app. 90000	0.66
whole OKT3 Fig.4	app. 75000	
F(ab´)2 OKT3 Fig.3	app. 16000	0.12
F(ab´)2 OKT3 Fig.4	app. 32000	
anti CD3 Diabody as claimed		0.00

Table 1: shows the amount of incorporated radioactive thymidine or dye absorbance, respectively, after incubating 10µg/mL F(ab´)$_2$ anti CD3 fragments or 10µg/mL of the whole OKT3 with PBMCs or 10µg/mL of the anti CD3 diabody according to the claimed invention. The starting values of the control buffer without antibody have been subtracted from the values measured at 10µg/mL.

From the data of Table 1 the percentage stimulation of F(ab´)$_2$ fragments and diabody according to the claimed invention compared to OKT3 is determined as follows:

Woodle et al. Fig. 3	OKT3 F(ab´)2 : whole OKT3 mAb	=	18 %
Woodle et al. Fig.4	OKT3 F(ab´)2 : whole OKT3 mAb	=	43 %
Experiment under item A)	OKT3 F(ab´)2 : whole OKT3 mAb	=	18 %
Experiment under item A)	Diabody (scFv$_6$) : whole OKT3 mAb	=	0 %

Thus, the F(ab´)$_2$ fragment stimulated a PBMC proliferation in the amount of 18% and 43%, respectively, compared with the stimulation by the whole monoclonal OKT3. In contrast, the diabody according to the claimed invention did not show any stimulation of PBMC proliferation.

Therefore, the data demonstrates that anti-CD3 F(ab´)$_2$ fragments, whether they were tested by Woodle et al. or in the present experiment, provide a significant stimulation of PBMC proliferation at concentrations where no significant proliferation is observed for the same quantity of a diabody according to the claimed invention.

It was unexpected that an anti-CD3 diabody according to the above-referenced patent application exhibited such a significant immunosuppression effect, and that such effect was superior over the closest prior art F(ab´)$_2$ fragments.

6. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 19th July, 2010

M. Little

Dr. Melvyn Little
Chief Scientific Officer
Affimed Therapeutics AG